Mass Spectrometry-based Lipidomic Approaches to Identify Lipid Markers of Inflammation

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Mass spectrometry (MS) in combination with liquid chromatography (LC), or LC/MS, is the preferred technique for lipidomic analyses. One of the most used applications of lipidomics is the search for molecular markers that can be used to signal specific activation states and that, ultimately, can be used in the early diagnosis of diseases. Our recent studies with monocytes/macrophages stimulated by inflammatory stimuli have revealed changes that result in the accumulation of unusual lipids. These changes are sometimes stimulus-specific and thus, could be regarded as specific markers of activation. Two examples are discussed below that resulted from the lipidomic analysis of arachidonate-containing phospholipids, and the analysis of the fatty acid composition of neutral lipids accumulating in cytoplasmic lipid droplets.

Transcription of the talk presented on Thursday October 27, 2016 at the Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Brazil, (Slide 1 – Title).

Long time ignored for being supposedly boring, lipids are gaining more and more acceptance as key molecules in physiology and pathophysiology. Even today, for many scientists and non-scientists, the word "lipid" takes them to inevitably think of these substances as simple sources of food energy or as structural components of membranes. This vision, in addition to limited, is erroneous, because lipids are a lot more than that; they are fundamental in physiology and pathophysiology because they regulate intercellular communication (Slide 2 - Lipids Are Key to Signaling). Today, it is firmly established that lipids connect the messages coming from external sources to the execution of specific responses by these cells. From a general point of view, we could classify the stimuli that use lipids to communicate messages in three groups, not mutually exclusive. In the first place, the stimuli that signal through changes in the intracellular calcium concentration. To this group belong most hormones and neurotransmitters. These stimuli activate a phospholipase C that is specific for inositol lipids that generates two messengers, one of lipid nature, diacylglycerol (DAG) and the other water-soluble, inositol trisphosphate (IP_3). Both messengers cooperate in a synergistic manner in the execution of cellular responses. Next, we have those stimuli that induce cell death, here to the left. These stimuli activate another enzyme of lipid metabolism, similar to the previous one, but this one hydrolyzes sphingomyelin to generate ceramide, that activates routes leading to cell death. Finally we have another group of stimuli that signal through the activation of phospholipase A_2, an enzyme that is slightly different from the previous ones in that it produces two messengers of lipid origin, with the peculiarity that both can act inside and outside the cells. The first of these is the lysophospholipid, which can be secreted and act on neighbouring cells, amplifying in this way the response. On the other hand, we have a free fatty acid, of great interest if it is arachidonic acid; the precursor of an enormous collection of compounds with potent biological activity with both physiological and pathophysiological regulatory effects: the eicosanoids, namely prostaglandins, leukotrienes etc, which are

involved in homeostatic maintenance of a large number of tissues and organs (Slide 3 – Eicosanoids in Physiology), and also participate in physiopathological processes, in particular in the inflammatory reactions, of which they are essential mediators (Slide 4 – Eicosanoids in Pathology). Inflammation is the response of an organism to an aggression of any kind. Inflammation is required to warn the organism that something bad is going on and a fix is needed. As I imagine that you know, all human disease consists of an inflammatory component, to a greater or lesser degree. (Slide 5 – Eicosanoids in Pathology). Indeed some of them diseases that kill more people in the world are of origin inflammatory (diabetes, cardiovascular disease) and that is initiated by inflammations poorly cured that, with the time is chronic and produce a huge damage in them tissues surrounding and in last instance lead to the death.

Well, as summary of all of the above we could make the following statement on the current vision of the lipids, where we emphasize that they are in fact the more important biological molecules since they regulate our vital functions and imbalances in lipid metabolism causes a large number of diseases. Therefore, if we want to cure these diseases, must know what lipids are involved and what is what make (Slides 6 & 7 – Current View of Lipids). For this mission, we have a very powerful tool, lipidomics. So what is lipidomics? There are many definitions, but we stick to te one provided by people (at the time) of the ICBL because it emphasizes the integrative aspect of this discipline (Slide 8 – What Is Lipidomics?). So, lipidomics is not only chemometrics; we need to put the findings in the appropriate biological context.

Our lab has been doing lipidomics for quite a long time now, always with a focus in signaling lipid in immunity innate and inflammation (Slide 9 – Lipidomics in Valladolid). In this sense it is important to note that a lipidome is made up of thousands of different molecules with very different chemical structure. This makes lipidomics inherently complex, because there is much structural variety. Therefore, attempting to unravel the full lipidome of a given cell system is complex and complicated, unless you have enormous resources, both money and trained personnel (Slide 10 – Lipid Categories). Those who cannot play that league, use the “Julius Caesar” strategy, i.e. “divide and conquer”. That means specioalize in a bit of the lipidome and work thoroughly on it. So we we have focused on arachidonic acid (AA), on thre directions: (i) the oxygenated derivatives, (ii) all fatty acid relatives of the omega-6 series and also omega-3 and omega-9, and (iii) the phospholipids into which arachidonic acid is esterified (Slide 11 – Lipidomics in Valladolid). This takes us to a key concept in AA regulation by phospholipase A_2 as shown before, which is that AA is not free in the cells and is produced "on demand", i.e. under stimulation to produce the oxygenated metabolites that are necessary each time. (Slide 12 – Role of Phospholipase A_2 in Arachidonic Acid Release). The rest of the time is "hidden" in the membrane phospholipids. In human cells AA is distributed almost exclusively in three kinds of phospholipids, PC red, green PE and PI in yellow (Slide 13 - Role of Phospholipase A_2 in Arachidonic Acid Release). This situation is actually more complex from a molecular point of view, because cells contain multiple enzymes with phospholipase A_2 activity, all of them being potentially capable of effecting the release of AA from phospholipids. The PLA_2s that participate in these processes can classified is in three families, c, s, and i, of which the $cPLA_2$ is the key enzyme, $sPLA_2$ is accessory, and $iPLA_2$ does not seem to be directly involved in releasing the AA but in keeping the AA pools withing phospholipid by acyl exchange (Slide 14 –Distinct Roles in Signal Transduction for Each of the Phospholipase A_2...). And things can get even more complicated if we take into account that AA is an intermediary of a seemingly futile cycle of deacylation/reacylation. The key here is that AA does not return to the phospholipid molecule from which it initially came out (Slide 15 – Free Arachidonic Acid Levels Depend on a Deacylation/Reacylation Cycle), because there are reactions of transacilacion between phospholipids where AA is transferred and therefore generates a specific distribution profile for each condition (Slide 16 – Arachidonic Acid Trafficking Among Phospholipids). In a nutshell, the homeostasis of the AA could be summarized as a tug-of-war between the $cPLA_2$ by pulling on one side and the enzymes of the reacylation and transacylation pathways on the other (Slide 17 –The Arachidonic Acid Tug-of-War). Thus, it is important to know which within each class of phospholipid molecular species is the AA and which and how much of each ais used during stimulation since this information can be very useful to study the

cellular regulation but also in identifying markers of different states of activation (Slide 18 – Lipidomics as a Useful Approach).

This is the experimental protocol utilized (Slide 19 – Lipidomic Profiling of Arachidonic Acid-containing Phospholipids). In these studies we use human monocytes and stimulate them with a typical innate stimulus, yeast-derived zymosan. This will activate the cells and will result in the activation of phospholipase A_2, which leads to the release of the AA, and the production of eicosanoids. This all results in the loss of AA in cellular phospholipids. Then we stop the reactions, extract the lipids and analyze AA-containing phospholipids, which is easy because they will give a signal of 303 m/z in fragmentation experiments. As a control we use a thing called pyrrophenone, which is an inhibitor of phospholipase A_2. Before moving on to show data, a small comment on the nomenclature of lipids (Slide 20 – Glycerophospholipids. Nomenclature). Do not forget to mention the ethers (Slide 21 – Glycerophospholipids. Nomenclature) and the plasmalogens (Slide 22 – Glycerophospholipids. Nomenclature). With this information, here we have what I call the arachidonome of human monocytes (Slide 23 –Arachidonic Acid-containing Phospholipids of Human Monocytes). This is already a 'diagnostic' result, it is already a lipid fingerprint of monocytes, because it presents unique characteristics that make it immediately identifiable as belonging to monocytes. For example, the high AA content in PE plasmalogens, and high content of AA in a single PI species. What we will do is to analyze the behavior of each them during cell activation. We would expect for them to go down with time. The time-courses of different kinds of phospholipids, PC are displayed (Slide 24 – Time-dependent Changes of Major AA-containing PC Species After Zymosan Stimulation), PI (Slide 25 – Time-dependent Changes of Major AA-containing PI Species After Zymosan Stimulation), and PE (Slide 26 – Time-dependent Changes of Major AA-containing PE Species After Zymosan Stimulation), emphasizing the appearance of species showing unusual behavior, i.e. increases. Studies with other stimuli indicate that one of them is specific of stimulus; these species could be regarded as markers of specific activation states, since they occur under certain conditions, but not for all (Slide 27 – What About Other Stimuli?). As a conclusion of these experiments is that, apart from their value as possible biomarkers, we could try to go a little further and try to study their function at the molecular level, what is the reason why cells make them (Slide 28 – Conclusions). A strategy to study this could be to isolate these lipids and introduce them in the cells to mimic a situation of activation. To get more on this, we will focus on PI(20:4/20:4) because it is an anionic phospholipid and as such, it can be transfectedm just like you would do with DNA or RNA (Slide 29 – Intracellular Delivery of Anionic Phospholipids). The protocol to study biological actions shown in the following figure (Slide 30 – Incorporation of PI(20:4/20:4) Into Cells). We focused on early responses such as the production of reactive oxygen metabolites. To the test the effect of PI(20:4/20:4) we studied whether the response was changed in cells loaded with this lipid (Slide 31 – PI(20:4/20:4) Regulates Superoxide Anion Production). We tried another response of innate immunity such as lysozyme secretion and observed a similar behavior (Slide 32 – PI(20:4/20:4) Regulates Lysozyme Release). All of this leads us to postulate that this lipid could regulate innate immune responses in macrophages (Slide 33 - 1,2-Diarachidonyl-sn-glycero-3-phosphoinositol).

Well, so far we have spoken of lipidomic analysis of phospholipids containing arachidonic acid; so, what happens with those who have no arachidonic? (Slide 34 – What about phospholipids without arachidonate?). To study this use a protocol identical to the shown previously, in fact is the same slide, but with an important variation. In this case we analyzed not only the effects of pyrrophenone, but also those of an inhibitor of $iPLA_2$-VIA, FKGK18, to analyze the possible contribution of this enzyme (Slide 35 – Lipidomic Profiling of Phospholipids Without AA). This figure shows an analysis by LC/MS of some of the most abundant species of PC without arachidonic and the effect of the inhibitors pyrrophenone and FKGK18 (Slide 36 - Major PC Species Without AA in Zymosan-stimulated Macrophages). Some of these species decrease with the activation, which indicates that they are hydrolysed. Pyrrophenone does not do anything, which is not is strange since this is an inhibitor of an enzyme that hydrolyzes lipids with arachidonic acid. However, FKGK18 completely prevents hydrolysis of some of these species. Examples where reversion is total include PC(32:0)

and PC(34:1), older species that could be identified in experiments of fragmentation as PC(16:0/16:0) and PC(16:0/18:1). Total reversal in other two minor species, PC(32:1) and PC(34:2), which were identified as PC(16:0/16:1) and PC(16:0/18:2) was also observed. Therefore, it is striking that four phospholipids identified as iPLA$_2\beta$ substrates during zymosan activation all contain palmitic acid (16:0) esterified at the sn-1 position. Actually when we measured lysolipid production in activated macrophages, palmitoyl-lysoPC was the major species produced and this production was strongly blocked by FKGK18, again suggesting that iPLA$_2\beta$ may be a major contributor to this accumulation (Slide 37 – Lysophospholipid Production in Zymosan-stimulated Macrophages). Note in contrast the other major lysolipid produced in the activated macrophages, stearoyl-lysoPC, whose levels are affected little by FKGK18 but strongly by pyrrophenone, suggesting a link of this species to cPLA$_2\alpha$. So in the end, as a conclusion of these experiments, in activated cells, iPLA$_2\beta$ displays some sort of specificity for PC molecules containing palmitic at the sn-1 position (Slide 38 – iPLA$_2$ May Have Preference for *sn-1* palmitoyl PC).

When we examined the inositol lipids we found a few of them that showed an unexpected behavior, as they increased instead of going down. However the increase was partially prevented by FKGK18 but not by pyrrophenone, suggesting that iPLA$_2\beta$ may be involved (Slide 39 – Minor PI Species Without AA in Zymosan-stimulated Macrophages). These species have in common that all possess a 16:1 fatty acid residue. And it is interesting to note that two of them, the two that increase the most, were not found in resting cells, suggesting that if the cells make them, they must have some biological function. So the question is, where does 16:1-PI come from? (Slide 40 – Where Does 16:1-PI Come from?). We measured all the phospholipids that have 16:1 and this is what we found (Slide 41 – 16:1-containing Phospholipids in Resting Macrophages). Most of the fatty acid is in PC, especially in one species, PC(16:0/16:1), while all other classes contained lesser amounts. Now we remind you that this PC species is one of those that I shown in the previous slide that was hydrolyzed by iPLA during activation to give rise to palmitoyl-lysoPC. So we measured the levels of the 16:1-containing phospholipids and found that PC(16:0/16:1) was the only that decreased and the ones that increased were the two preexisting PI molecules plus the 2 newly-formed (Slide 42 – 16:1-containing Phospholipids in Stimulated Macrophages). So, it is likely that much of the 16:1 fatty acid accumulating in PI comes from PC in a reaction that is sensitive to FKGK18 and thus probably mediated by iPLA$_2\beta$ (Slide 43 – Where Does 16:1-PI Come from? PC(16:0/16:1). One problem with this explanation is that the amount lost from PC is generally less than the amount gained by the PIs. Thus there is possibly a second source for 16:1 for PI. Because of this, we extended our analysis of distribution of 16:1 among lipid classes, also to neutral lipids, and we did this by GC/MS. We were surprised to find that, depending on the lipid class measured, there were two peaks of 16:1 (phospholipids) or only one (neutral lipids) (Slide 44 – Two 16:1 Isomers in Macrophages). Comparison with commercial standards indicated that one was 16:1n-7 or palmitoleic acid proper and the other could either be 16:1n-10 (sapienic acid) or 16:1n-9 (Slide 45 – Two 16:1 Isomers in Macrophages). We made the DMOX derivative and analyzed it by electron impact MS. Confirming it was actually 16:1n-9, a relatively unusual fatty acid (Slide 46 – The Second Isomer is 16:1n-9).

What is the metabolic origin of 16:1n-9? (Slide 00 – Metabolic Origin of 16:1*n-9*). Since there are no Δ^7 desaturases in mammalian cells, a likely possibility is that it derives from oleic acid (18:1*n-9*). Incubation of the cells with oleic acid increased the levels of 16:1n-9 (Slide 00 – Enrichment with Oleic Acid Increases Cellular 16:1n-9 Levels). This question was analyzed more directly by using deuterated oleic acid (Slide 00 – Fatty Acid Methyl Ester (FAME) Fragmentation Spectra). There are clear differences between the spectra of native and deuterated oleic acid, and incubation of the cells resulted in the appearance of a second deuterated fatty acid with the expected mass of 16:1n-9 and characteristic fragments consistent with it being 16:1n-9 (Slide 00 – Fatty Acid Methyl Ester (FAME) Fragmentation Spectra). Finally, if oleic acid is the actual precursor of 16:1n-9 this has to coccur via β-oxidation; etomoxir, an inhibitor of this route, blunts 16:1n-9 accumulation (Slide 00 – Effect of Etomoxir on 16:1n-9 Accumulation). So, as a summary of these data it seems that strikingly, during

activation, immunoinflammatory stimuli activate both biosynthesis and discrete metabolism of fatty acids to generate diversity (Slide 00 – Lipid Inflammatory Signals Regulate Cellular Lipid Metabolism).

As stressed before, there is a fundamental difference in the distribution of these two isomers among lipid classes, the n-9 isomer localizes in both phospholipid and neutral lipids, whereas n-7 only appears in phospholipid (Slide 47 – Distribution of 16:1 Isomers Between Lipid Classes). This distribution of n-9 is quite peculiar since no other fatty acid distributes similarly (Slide 48 – Distribution of 16:1 Isomers Between Lipid Classes). When we analyzed the distribution of n-9 in zymosan-treated cells, we found that it increases in all lipid classes but the highest increases are in neutral lipids (Slide 49 – Distribution of 16:1 Isomers Between Lipid Classes).

The unique distribution of 16:1n-9 among cellular lipids and the finding that its levels are increased during cellular activation suggest a specific biological role for this unusual fatty acid. To study this question, we prepared cells enriched in this fatty acid by incubating them with 10 μM 16:1n-9 for 2 h in serum-free medium. This procedure results in the cells taking up the fatty acid and preferentially accumulating it in neutral lipids, in a similar manner as if they had been previously activated with a receptor-directed stimulus (Slide 50 – Assessing the Biological Effects of 16:1n-9). Then, the cells were stimulated with LPS and the effects on the expression of a number of proinflammatory genes was investigated (Slide 51 – 16:1n-9 Possesses Anti-inflammatory Properties *in vitro*). Cells enriched in 16:1n-9 showed significant decreases in the expression of all genes tested, and such decreases were generally comparable to those found in the 22:6n-3-treated cells. 16:1n-9 was significantly more potent than 16:1n-7 for all genes tested; 16:1n-7 had significant effects only in two of them, Tnf and Nos2. These data show that 16:1n-9 has a spectrum of biological activity that is clearly distinguishable from that of 16:1n-7. We also conducted experiments with mice (Slide 52 – 16:1n-9 Possesses Anti-inflammatory Properties *in vivo*). In these experiments, the fatty acid was administered i.p. to mice 1 h before i.p. injection of LPS for 6 h. Afterward, the animals were sacrificed, peritoneal cells were harvested, cell samples matched by protein content, and the expression levels of Il6 were studied. Both 16:1n-9 and 22:6n-3 inhibited Il6 gene expression by the peritoneal cells isolated after the LPS challenge. Analysis of serum IL-6 protein confirmed a strong decrease in the amount of circulating IL-6 protein in the 16:1n-9-treated mice. Unexpectedly, IL-6 protein levels in serum from 22:6n-3 treated cells were no different from those in serum from control untreated animals.

I will finish my talk by emphasizinh once again the usefulness of a lipidomics approach to find markers of activation and new molecules with roles hitherto unknown in inflammation, such as PI(20:4/20:4) and 16:1n -9, keeping always in mind a sentence that I had introduced eary in my talk: "if we are going to solve..." (Slide 53 – Lipid Markers of Activation). And in that regard, let me finish with a small lesson on history of medicine by remembering that the type of studies and information we get from lipidomics complies with something that a man prophesized more than 100 years ago, Johan Ludwig Thudichum (Slide 54 – The Need for Metabolomics), and what science thought about Thudichum shortly after his death (Slide 55 – Thudichum). Just a couple of minutes more to thank my collaborators and sources of support (Slide 56 – Acknowlegments).

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